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Complementation of Defective Reovirus by ts Mutants

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Defective reovirions lacking the largest (L_1) of the normal 10 genomic segments grow only in association with helper reovirus. Because of the similarity in properties of defective and infectious virions, separation of the two populations by physical methods has been unsuccessful. Controlled digestion of purified virus removes the outer capsomeres of the virions. The resulting core particles containing the viral genome have a buoyant density of 1.43 g/ml if derived from infectious virions and of 1.415 g/ml if they originate in defectives, and this difference permits ready separation of the two types of cores. With the purpose of obtaining a pure population of defective virions, L cells were co-infected with defective cores and a class E temperature-sensitive mutant which has a mutation in an early function. After three serial passages at the permissive temperature (31 C) to build up the defective population, a fourth passage was made at 39 C, the nonpermissive temperature. The virus purified from this passage was predominantly defective; it contained practically no E mutant and had a low background of wild-type virus. Complementation was thus asymmetric; the L_1 function required for growth of defective virus was supplied by the E mutant and is thus a *trans*-function, while defective virus did not complement the E mutation which is thus in a *cis*-acting function. Defective virions were indistinguishable from infectious virions except for the absence of the L_1 genomic segment in the defectives. Such defective virions could be complemented at 39 C by class A and B temperature-sensitive mutants, both of which have lesions in late functions.

In common with many other mammalian viruses (6), populations of reovirus frequently contain defective particles. These defective particles grow only in association with infectious helper virus and it has been shown that they lack the largest (L_1) of the 10 double-stranded RNA (dsRNA) segments of the infectious viral genome (11, 13).

With the object of studying the biological properties of defective virions, we have made a number of attempts by various physical methods to separate them from infectious particles. These efforts have been consistently unsuccessful because of the very minor differences between the two types of particles in any of the physical properties examined. In fact, the presence of defective particles in a population of reovirus is most simply demonstrated by controlled digestion of the purified virus with chymotrypsin. The outer layer of viral capsomeres is thereby removed (8, 17) leaving the protein core which contains the dsRNA genome. During subsequent isopycnic centrifugation of the enzyme-treated virus in CsCl, defective cores band at a considerably lower density than cores from infectious virions (11, 13) and can thus be separated from them. Cores obtained in this way from infectious virions still retain an ability to infect cells at a level approximately 10^{-4} that of an equivalent number of infectious virions. The infectivity of defective cores is at most 10^{-7} that of the viral population from which they are derived as will be shown. In the present work we have used these defective cores to develop a method for producing a population of defective virions based on the following proposition.

Since the growth of defective particles depends on the helper function of infectious wild-type virus, the same helper function ought to be provided by one or other temperature-sensitive (*ts*) mutant of reovirus at the nonpermissive temperature where the *ts* mutant itself would not grow. A number of *ts* mutants of reovirus have been isolated and classified into seven groups by genetic recombination and complementation tests (2-4, 7, 15). In the present work we have added defective cores and the E class mutant to L cells in the hope that sufficient cores would be taken up by the cells to grow in association with the mutant at the permissive temperature. It is shown that such growth of defective particles occurs, and that subsequent passage of the progeny at the non-

permissive temperature results in an almost pure population of defective virions.

MATERIALS AND METHODS

Cells and virus. L cells were grown in suspension culture in minimum Eagle medium supplemented with 5% fetal calf serum.

We have used two different wild type strains of type 3 reovirus. The first strain has been in our laboratory for a number of years; it was isolated originally as a single plaque from the Dearing strain of reovirus obtained from P. Gomatos (Sloan Kettering Institute). After 7 to 8 serial passages at high multiplicity of infection (MOI), this strain has invariably given rise to defective virions which have the largest (L_1) of the 10 genomic segments deleted (11, 13). Infectious virions of this strain or cores derived from them by chymotrypsin digestion (8, 13) will be designated R_1. Defective virions or cores derived from this strain will be designated R_1d (L_1) or, for present purposes, R_1d since there is no need to distinguish between deletions of L_1 and other segments in this paper. The second wild-type strain was obtained from B. N. Fields (Albert Einstein School of Medicine) and will be designated R_2. In our hands this strain has not yet given rise to defective virions, although we have not persisted in carrying it to high passage levels. It was from the R_2 strain that Cross and Fields (2) and Fields et al. (3, 4) isolated the seven classes of *ts* mutants that are termed A, B, C, D, E, F, and G. We are grateful to B. N. Fields for supplying us with prototypes of these mutants. In the present work we have employed mutants of classes A, B, and E. With the suggested nomenclature these would be referred to formally as R_2A(201), R_2B(352), and R_2E(320); the numbers in brackets being those given to the particular mutants by Fields and Joklik (4). This terminology can be readily expanded if other wild-type strains that are defective turn up, if different deletions are found, or if mutants of type 1 and type 2 virus come into use. It should be mentioned that we have isolated a number of *ts* mutants from our R_1 strain that recombine with one or other of the Fields prototype mutants, although they have not yet been classified into groups. The two wild type strains are, therefore, closely related to each other.

The plaque assay was carried out as described (14). Permissive temperature was 31 C and nonpermissive temperature 39 C.

Buffers, chemicals and isotopes. STE buffer (0.01 M) contains 0.01 M NaCl, 0.05 M Tris-chloride (pH 7.4), and 0.001 M EDTA. STE buffer (0.3 M) contains 0.3 M NaCl in place of 0.01 M NaCl. TE buffer contains 0.01 M Tris-chloride (pH 7.4) and 0.001 M EDTA. TNM buffer contains 0.25 M NaCl, 0.01 M Tris-chloride (pH 8), and 0.01 M 2-mercaptoethanol. PBS (pH 7.3) contains 0.13 M NaCl, 2.7×10^{-3} M KCl, 8.2×10^{-4} M Na_2HPO_4, 1.5×10^{-3} M KH_2PO_4, 9.1×10^{-4} M $CaCl_2$, and 5×10^{-4} M $MgCl_2$. SSC contains 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.4. [5-^{3}H]uridine (25 Ci/mmol), [2-^{14}C]uridine (50 mCi/mol), L-[^{35}S]methionine (100 Ci/mmol), and L-^{3}H-labeled amino acid mixture in 0.1 N HCl solution were obtained from New England Nuclear.

Preparation of purified virus and labeled virus. Suspension cultures of L cells were centrifuged at 600 $\times$ *g* for 10 min and the cells were suspended in minimum Eagle medium at a concentration of 10^7 per ml. Purified virus was added to a MOI of 20 PFU/cell, or as otherwise specified for particular experiments. After an adsorption period of 6 h, during which time the culture was stirred with a magnetic bar, the cells were centrifuged and suspended at 5×10^5 per ml in minimum Eagle medium containing 2% fetal calf serum. The time of suspension was taken as zero time postinfection. Infected cultures were stirred magnetically at 31, 37, or 39 C depending on the purpose of the experiment, and the cells were harvested at corresponding times of 40, 20, or 12 h. At these times when 50 to 80% of the cells had lysed, the cultures were centrifuged at 600 $\times$ *g* for 20 min and the sediment, containing most of the virus, was suspended in TNM buffer. Homogenization of this suspension with Freon 113 and sedimentation of the virus into a CsCl cushion has been described (20). The band of virus was collected from the cushion, dialyzed against SSC, and the virus was centrifuged into a pellet at 27,000 rpm for 60 min in an SW27.1 Beckman rotor. After suspending the pellet by sonically treating it with 2 ml of SSC buffer the suspension was layered over a 20 to 40% linear sucrose gradient and centrifuged in the SW27 rotor for 60 min at 25,000 rpm. Most of the virus appeared in a narrow band near the center of the tube; it was removed, dialyzed against SSC for 3 h, and centrifuged through a preformed gradient of CsCl (1.32 to 1.43 g/ml) for 100 min at 26,500 rpm in the SW27 rotor. Finally, the band of virus was isolated and dialyzed against SSC. When stored at -70 C this purified virus was stable for several months. To prepare labeled virus, actinomycin D (0.5 μg/ml) was added to a culture at zero time postinfection along with 1 μCi of [^{3}H]uridine per ml, 0.02 μCi of [^{14}C]uridine per ml, 5 μCi of ^{3}H-labeled amino acid mixture per ml, or 1 μCi of [^{35}S]methionine per ml in methionine-free medium. Labeled virus was purified as described above. To calculate the number of particles in a purified virus preparation the relationship 2.1×10^{12} particles per optical density $(OD)_{260}$ was used (17). On the average, R_2 virus contained 50 particles per PFU; R_2A(201), 50 to 80 particles per PFU; R_2B(352), 50 to 80 particles per PFU; R_2E(320), 100 to 150 particles per PFU. For reovirus cores the value used was $6.5 \times 10^{12}/OD_{260}$ whether the cores were defective or not. This value for cores was obtained by measuring the OD_{260} of a suspension of cores from a known number of R_2 virions; the use of the same value for defective cores introduces a small error which has been neglected in the present work.

Preparation of viral cores. Purified virus, 10^{12} to 4×10^{12} particles per ml, in 0.05 M Tris-hydrochloride buffer (pH 8.0) was treated with 100 μg of chymotrypsin per ml (Worthington Biochemicals Corp.; crystallized five times) for 90 min at 37 C and layered over a preformed CsCl gradient (1.35 to 1.46 g/ml in 0.01 M STE buffer). Centrifugation was for 6 h at 37,500 rpm at 4 C in the Beckman SW40 rotor when the viral

cores had banded at their equilibrium densities. Fractions were collected and assayed for radioactivity and OD_{260}.

Extraction of dsRNA and polyacrylamide gel analysis. Purified virus was dialyzed against 0.3 M STE buffer. Sodium dodecyl sulfate (SDS) was then added to a concentration of 0.5% (wt/vol) and dsRNA was extracted with water-saturated phenol at room temperature. Three volumes of ethanol were added to the aqueous extract and the mixture was left at −20 C for 18 h to permit the RNA to precipitate. After centrifugation, the precipitate was washed three times with 70% ethanol and then analyzed by electrophoresis on 5% polyacrylamide slab gels (18, 19). The dimensions of the slab were 20 by 15 by 0.15 cm. Gels were formed with 5% acrylamide, 0.12% N,N'-methylene bisacrylamide in 0.25 M Tris-chloride (pH 8.3), and 0.04% *N,N,N',N'*-tetramethylethylenediamine, and polymerization was catalyzed with 0.6% ammonium persulfate. Fifty microliters of each sample, in which three of four small crystals of sucrose were dissolved, was placed into sample wells 0.8 cm wide. Electrophoresis was carried out at 50 mA for 50 h in a buffer containing 0.05 M sodium acetate, 0.001 M EDTA, and 0.04 Tris-chloride (pH 7.4). Gels were then placed on a piece of Whatman no. 5 paper, dried in vacuo over boiling water, and autoradiography was carried out with Kodak X-ray film. Autoradiograms were exposed for 1 to 5 days: dsRNA samples containing 10,000 cpm of ^{14}C yielded visible bands in 24 h. Regions of the gel containing the radioactive bands were cut into 1 mm slices, solubilized in 30% H_2O_2, and the radioactivity was determined by liquid scintillation counting.

Analysis of reovirus proteins on polyacrylamide gels. Purified virus, labeled in its protein with either ^3H-labeled amino acid mixture or [^{35}S]methionine, was adjusted to 2% concentration with each of SDS, 2-mercaptoethanol, and 0.1 M with phosphate buffer (pH 7.2), and heated in a boiling water bath for 2 min to dissociate the proteins. The sample was made 10% in sucrose and analyzed on a 10% polyacrylamide gel (15 cm in length and 0.6 cm in diameter) prepared in 0.1 M phosphate buffer (pH 7.2), 6M urea, 0.1% SDS, 0.02 M EDTA, 0.26% bisacrylamide, 0.1% *N,N,N',N'*-tetramethylethylenediamine, and 0.08% ammonium persulfate. Electrophoresis buffer consisted of 0.1 M sodium phosphate (pH 7.2), 0.1% SDS, and 0.01 M EDTA and electrophoresis was carried out for 40 h at 6 mA per tube. Gels were cut into 1 mm slices, solubilized in 30% H_2O_2, and the radioactivity was determined by liquid scintillation counting.

RESULTS

Preparation of Defective Cores. For the present work a single plaque was selected from the R_1 strain of virus, described under Materials and Methods, and serially passaged a total of 12 times in L cells. Undiluted lysates were used between the first and third passages, and subsequent passages were carried out at multiplicities of approximately 20 PFU/cell. Twelfth passage virus was frozen at −70 C and used as stock virus.

At various passage levels, the ratio of complete to defective virus was determined. This was done by labeling the virus during a given passage with [^3H]uridine, purifying the virus, digesting it with chymotrypsin, and banding the resulting cores by isopycnic centrifugation in a preformed gradient of cesium chloride. The ratio of complete R_1 cores to defective R_1d cores remained approximately constant after the ninth passage. Figure 1 shows the radioactivity profile obtained in such an analysis during the 13th passage, i.e., the first passage of stock virus. R_1 cores band at a density of 1.43 g/ml and R_1d cores at 1.415 g/ml (13), and the ratio is approximately 1:5. The radioactivity at the top of the gradient represents the low molecular weight oligonucleotides known to be present in reovirions and released by chymotrypsin digestion (8).

Stocks of defective cores were obtained by

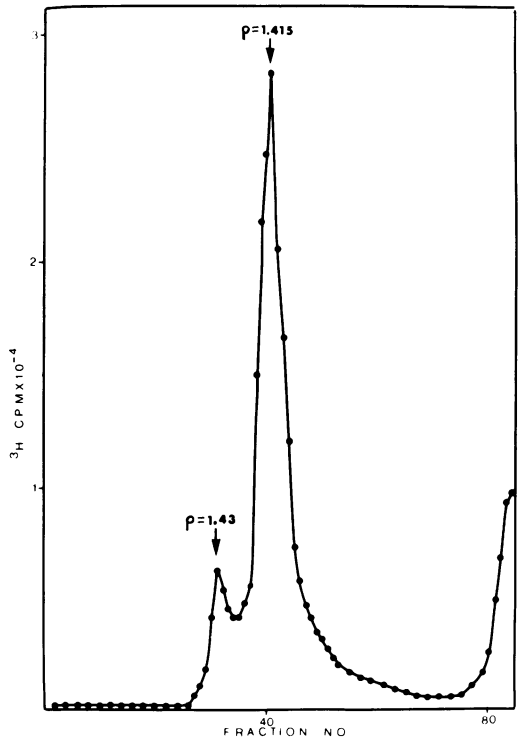

FIG. 1. *Sedimentation of chymotrypsin-treated reovirus (R_1) in a CsCl gradient. Purified [^3H]uridine-labeled, 13th passage R_1 virus (1 OD_{260}/ml) was digested with chymotrypsin and sedimented through a preformed CsCl gradient, as described in Materials and Methods. Direction of sedimentation is from right to left.*

centrifuging the cores obtained from approximately 10 OD_{260} units of purified, unlabeled virus to equilibrium in a cesium chloride gradient. Two sharp, well separated bands representing R_1 cores and R_1d cores could be seen. The upper band of R_1d cores was removed with a syringe by side puncture of the tube and dialyzed against PBS. Defective cores were stored at 4 C and used for complementation experiments within 24 h of their preparation. The infectious titer of defective cores was approximately 10^{-7} that of an equivalent number of R_1 virus particles.

Analysis of viral yield from L cells coinfected with defective cores and a ts mutant. The main purpose of these experiments was to determine whether *ts* mutants of R_2 virus could provide a helper function for the growth of defective virus at the nonpermissive temperature, i.e., whether complementation would occur. Most of the work was carried out with the R_2E mutant as helper; this virus has a mutation in a very early function and there is practically no synthesis of viral dsRNA in cells infected with it at 39 C (2, 7). Since defective virions were unavailable, the initial co-infection of cells had to be carried out with R_1d cores, which could be obtained in a high degree of purity, and the R_2E mutant. The critical issue was whether sufficient R_1d cores would enter the cells and be helped by R_2E virus to provide a population of R_1d virions during the first passage at 31 C. If so, the yield of defective virions ought to be increased by subsequent passages at 31 C. This procedure should provide a mixed population of R_2E and R_1d virions that could then be tested for mutual complementation by infecting cells with it at 39 C. The experiments described below provide the details of procedure and indicate that the basic suppositions were correct.

L cells in suspension were co-infected with purified R_2E mutant (15 PFU/cell) and R_1d cores (5,000 particles per cell). Approximately 80% of R_2E mutant and 10 to 15% of R_1d cores were adsorbed in 1 h at room temperature at a cellular concentration of 5×10^6 per ml. After the adsorption period the cells were diluted to 5×10^5 per ml and magnetically stirred at 31 C for 40 h. At this time, approximately half the cells showed cytopathogenic effect. The culture was sonically treated to complete the lysis and then passaged undiluted into another suspension culture of L cells. The procedure was repeated again to give the third passage lysate which was then plaque titered at 31 C, and subjected to the following tests for growth of defective virions.

Two cultures of L cells were infected with this third passage lysate at a MOI of 5 PFU/cell. One of these cultures was placed at 31 C, the second at 39 C, and [^{3}H]uridine was added to both cultures to label the progeny virus during its growth. In the remainder of this section these cultures will be referred to as 31 C culture and 39 C culture. The viral yield from each culture was purified and the plaque titers were determined at 31 C and 39 C. The numbers of viral particles per PFU at 31 C were also determined for the two purified virus preparations and the results are shown in Table 1 (rows 1 and 2). For the sake of comparison, similar parameters are shown in Table 1 for a purified preparation of R_2E mutant grown in L cells at 31 C (MOI equals 5 PFU/cell, row 3) for a purified preparation of wild-type R_2 virus grown at 31 C (row 4) and for R_1d cores.

Several points can be made about the results in this Table. (i) Whereas the yields of purified virus particles from the 31 C culture (row 2), the R_2E infection (row 3), and the R_2 virus infection (row 4) were approximately the same, about 10^5 particles per cell, the yield from the 39 C culture (row 1) was less than one quarter this amount. This lower figure, nevertheless, still represents a very considerable multiplication of viral particles at 39 C. (ii) Only one in 5×10^6 particles obtained from the 39 C culture were

TABLE 1. *Assays of virus preparations obtained under different conditions*

Sample	Yield of purified virus (particles/cell)[a]	PFU/OD_{260}		Particles/PFU at 31 C in purified virus
		31 C	39 C	
Purified virus from 39 C culture	2.5×10^4	4.2×10^5	1.1×10^5	5×10^6
Purified virus from 31 C culture	1.1×10^5	7.5×10^9	2.5×10^5	275
Purified R_2E virus grown at 31 C	1.1×10^5	2.1×10^{10}	6.9×10^5	100
Purified R_2 virus grown at 31 C	8.4×10^4	4.2×10^{10}	3.3×10^{10}	50
R_1d cores		$<10^3$	$<10^3$	$>10^9$

[a] Calculated from the OD_{260} determined on the total amount of purified virus recovered from 5×10^8 cells in a culture volume of 1 liter.

infectious when titered at 31 C (row 1, column 5) compared to one infectious particle per 50 to 275 particles in the other three cultures. Thus, the vast majority of particles obtained from the 39 C culture were not infectious. (iii) Considering the ratios of the plaque titers at 39 C and 31 C (columns 3 and 4) the expected ratio of approximately 3×10^4 was obtained for the R_2E mutant. A similar result was seen for the virus from the 31 C culture (row 2) suggesting that most of this virus was R_2E mutant. The ratio of unity was obtained for wild-type R_2 virus as expected. A quite different result was seen with virus from the 39 C culture (row 1) in that the small amount of infectious virus present gave essentially the same titers at permissive and nonpermissive temperatures. The infectious virus present in this preparation was apparently wild type and not R_2E mutant.

From these results we concluded that the R_2E virus grew readily in the 31 C culture. On the other hand, while there was extensive multiplication of noninfection particles in the 39 C culture, there was no co-incident growth of R_2E mutant.

To get more information about the nature of the particles formed in the 31 C and 39 C cultures, advantage was taken of the ^{3}H-label in the purified viruses to determine their buoyant densities in CsCl before and after treatment with chymotrypsin. A portion of each of the two preparations was mixed with purified ^{14}C-labeled R_2 virus to act as a density marker and centrifuged in a gradient of CsCl. Figure 2 shows the results obtained with virus obtained from the 39 C culture; results with the 31 C virus were similar. Virus particles formed under both permissive and nonpermissive conditions had approximately the same buoyant density as the marker virus.

Another portion of each of the two preparations was digested with chymotrypsin to convert the virions to cores and then centrifuged in a CsCl gradient. Results with virus grown at 31 C are shown in Fig. 3. A small peak of ^{3}H at the density of R_2 viral cores (ρ equals 1.43) represents growth of R_2E mutant. There is a large trailing peak of ^{3}H at the density of R_1d cores which presumably represents the multiplication of R_1d virions in the cells concurrently with the R_2E mutant. However, results with the 39 C virus in Fig. 4 show that the peak of R_2E cores was absent indicating lack of growth of R_2E virus at 39 C, in agreement with Table 1. Nevertheless, the large peak at the R_1d cores density in Fig. 4 shows that the defective virions multiplied at 39 C, their growth having been complemented by the R_2E mutant.

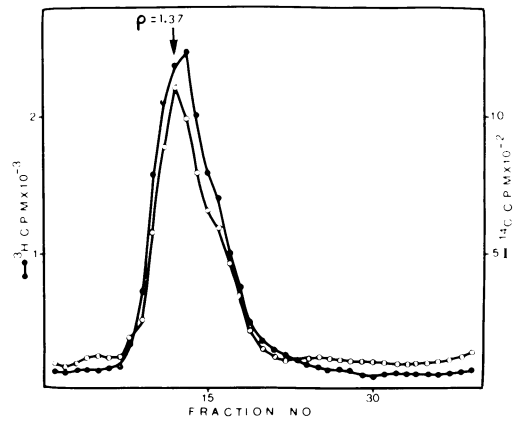

FIG. 2. *Isopycnic centrifugation of the ^{3}H-labeled virus obtained by infection of cells at 39 C with the third passage progeny from the mixed infection of R_2E virus and R_1d cores. The ^{3}H-labeled virus was mixed with ^{14}C-labeled R_2 virus as a density marker, solid CsCl was added to a density of 1.36 g/ml, and centrifugation was for 24 h at 40,000 rpm at 4 C in an SW50 rotor.* (○) *^{14}C-labeled R_2 virus;* (●) *^{3}H-labeled virus.*

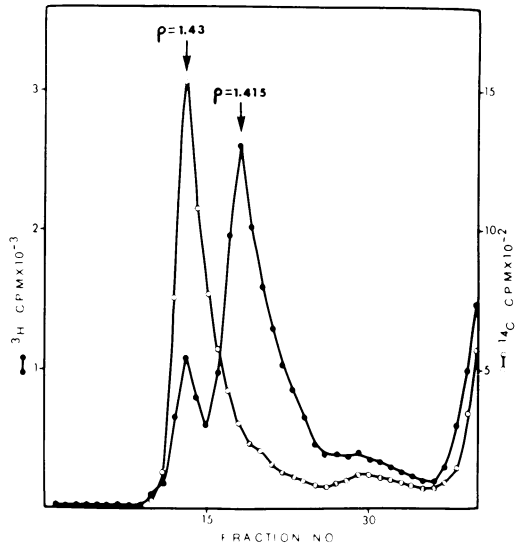

FIG. 3. *Sedimentation in CsCl of cores obtained from the fourth passage 31 C progeny of the mixed infection of R_2E virus and R_1d cores.* (○) *cores obtained from ^{14}C-labeled R_2 virus was added as a density marker;* (●) *^{3}H-labeled cores from the progeny of the mixed infection.*

As a control experiment to find whether the *ts*-mutant virus gave rise to defectives when passaged alone, cells were infected with 20 PFU/cell of R_2E at 31 C. The lysate from this culture was sonically treated and serially passaged three times in L cells at 31 C, just as had

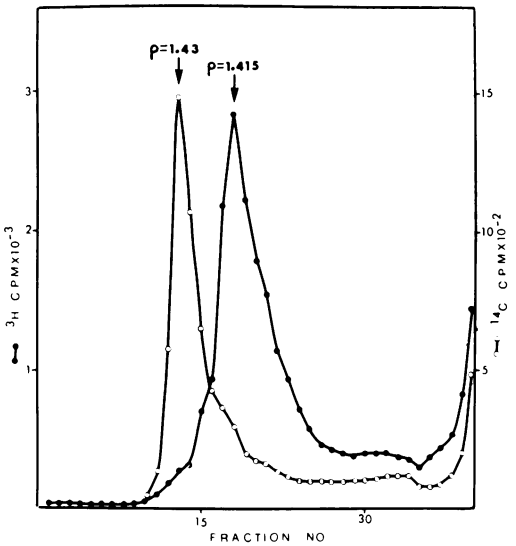

FIG. 4. *Sedimentation in CsCl of cores obtained from the fourth passage 39 C progeny of the mixed infection of R_2E virus and R_1d cores.* (○) *cores obtained from ^{14}C-labeled R_2 virus was added as a density marker;* (●) *^3H-labeled cores from the progeny of the mixed infection.*

been done during the mixed infection with R_2E virus and R_1d cores. Virus from the final lysate was used to infect cells (MOI equals 20) at 31 C in the presence of [^{3}H]uridine, the viral progeny of this infection was purified, digested with chymotrypsin, and centrifuged in a CsCl gradient. As shown in Fig. 5, all the ^{3}H-labeled R_2E cores banded with ^{14}C-labeled R_2 marker viral cores. Thus, no defective virus was derived from R_2E mutant under the conditions used in the mixed infection experiment.

These results are interpreted to mean that during the first passage at 31 C, R_1d cores entered the cells and grew in association with R_2E virions. During the succeeding passages at 31 C, the population of R_1d virions increased to the point where they comprised upwards of 70% of the total viral yield (Fig. 3). When cells were infected at 39 C with this mixed population of R_2E and R_1d virus, growth of the defective virions was complemented by the R_2E virus, but there was no reverse complementation and R_2E virus did not grow. The small amount of infectious virus found in this 39 C culture (Table 1) was wild type, perhaps derived from revertants of the R_2E mutant.

Analysis of the dsRNA in R_1d virions. The following experiment was done to ensure that the genome of defective virus had maintained its integrity during passage with the R_2E mutant. Cells were infected at 39 C with the third passage mixture of R_2E and R_1d viruses described in the previous section. The virus was labeled during its growth with [^{3}H]uridine, purified, and its dsRNA isolated. This dsRNA was mixed with ^{14}C-labeled marker dsRNA from R_2 virus and analyzed by electrophoresis on a polyacrylamide gel. The results were similar to those previously described for defective cores (11, 13); the usual 10 segments of dsRNA were found in R_2 virus, but no L_1 segment could be detected in the R_1d virus.

This result serves two purposes: it shows that the distribution of segments in R_1d virus is not changed by growing in association with R_2E virus, it supports the conclusion drawn from Fig. 4 and Table 1 that the virus obtained by this complementation is practically a pure population of R_1d (L_1) virus.

Analysis of the proteins in R_1d virions. While the similar buoyant densities of infectious and defective virions (Fig. 2) suggested that the latter virus contained the full complement of capsid proteins, this question was further investigated in the following way. Cells were infected at 39 C with the third passage mixture of R_2E and R_1d virus, as described in the two preceding sections. The virus was labeled during its growth with [^{35}S]methionine, purified, and mixed with purified R_2 virus which had been labeled in its protein with

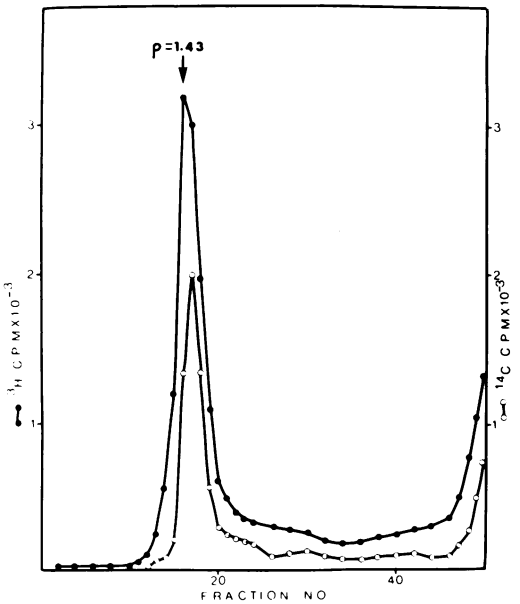

FIG. 5. *Sedimentation in CsCl of cores obtained from the fifth passage of R_2E mutant at 31 C.* (○) *cores obtained from ^{14}C-labeled R_2 virus was added as a density marker;* (●) *^3H-labeled cores obtained from the fifth passage R_2E virus.*

^{3}H-labeled amino acid mixture. The viral proteins were dissociated with SDS and analyzed by electrophoresis on a polyacrylamide gel with the results shown in Fig. 6. There was no essential difference between the proteins of the infectious and defective virions.

Figure 7 shows an electron micrograph of defective virions and there is no difference in the appearance or dimensions of these particles from those of infectious virus.

Rate of adsorption of defective virions. Figure 8 provides the results of an experiment in which the adsorption of ^{3}H-labeled R_2 virions and ^{35}S-labeled defective virions to L cells was measured. Both populations adsorbed at the same rate. These results provide further evidence for the integrity of the capsids of R_1d virions, as well as a practical measure of their adsorption rate for use in later experiments.

Complementation of R_1d virions by ts mutants R_2A and R_2B. In a preliminary experiment to determine whether other classes of *ts* mutants would complement defective cores, cells were co-infected with R_1d cores and either of the mutants R_2A(201) or R_2B(352). Two further serial passages were made, as described for the R_2E mutant. On a fourth passage at 39 C, growth of R_1d virions was found showing that both mutants had complemented the defective virus. However, this becomes a tedious procedure if one wishes to test a number of mutants for complementation of defectives. If the population of R_1d virions derived from growth with R_2E at 39 C could be used directly in the complementation test, the procedure would be considerably shortened and be more easily controlled as far as relative MOI's of defective virions and mutants are concerned. Such a test is described below.

Cells were co-infected with R_1d virions and mutant R_2B(352) at 39 C, and labeled with [^{3}H]uridine during viral growth. The resulting virus was purified, mixed with ^{14}C-labeled R_2 virus as a marker, digested with chymotrypsin, and then centrifuged in a CsCl gradient. A large peak at the R_1d core density (Fig. 9) shows that the growth of R_1d virus was complemented by R_2B mutant as expected, although there was apparently little growth of the mutant itself. Similar results were obtained with R_2A(201). Clearly, the R_1d virions will be useful in determining complementation between a variety of reovirus mutants and the defectives.

DISCUSSION

The practical upshot of this work is that we have been able to isolate a practically pure population of defective reovirions lacking the largest (L_1) of the 10 segments of the wild-type viral genome. As shown in Table 1 (row 1), the number of infectious particles in a purified preparation of defective virus was approximately 10^{-5} that of an equivalent number of *R*E or *R* virions. Apart from the deleted segment, the defective virions seem to be indistinguishable from infectious virus in that they have the same complement of capsid proteins, the same buoyant density in CsCl, adsorb to L cells at the same rate, and have approximately the same content of transcriptase per virion (unpublished results).

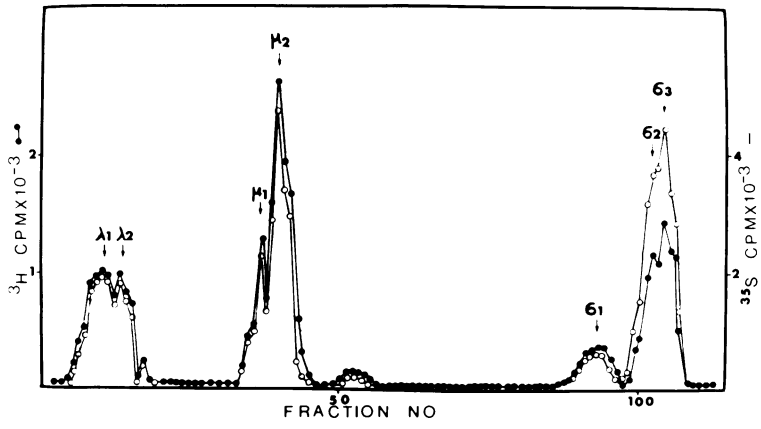

FIG. 6. *Analysis by electrophoresis on polyacrylamide gels of the capsid proteins obtained from the fourth passage 39 C progeny of the mixed infection of R_2E virus and R_1d cores. The virus was labeled with [^{35}S]methionine during its growth, purified, and mixed with purified R_2 virus that had been labeled with ^{3}H-labeled amino acid mixture.* (○) *[^{35}S]methionine proteins from the progeny of the mixed infection;* (●) *proteins from ^{3}H-labeled R_2 virus.*

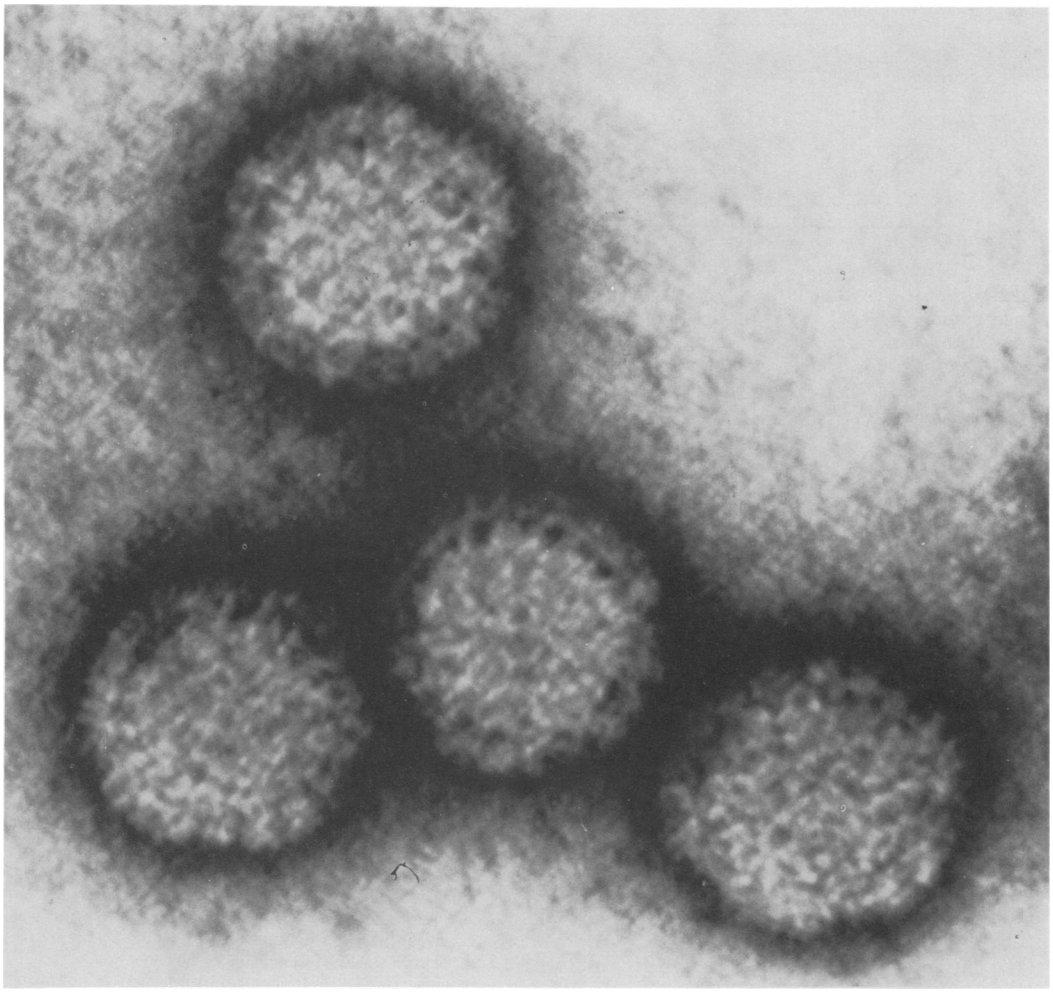

FIG. 7. *Electron micrograph of defective virions obtained through complementation with the R_2E mutant. Stained with 2% uranyl acetate.* ×*623,000.*

A variety of biochemical experiments with defective virus now become feasible. For example, we have found that in cells infected with R_1d virus there is no synthesis of viral dsRNA (unpublished results). Clearly the L_1 genomic segment controls an early function in viral replication. Since only four of the 10 segments of the viral genome are transcribed when protein synthesis, and consequently dsRNA synthesis, is blocked from the time of infection (10, 12, 20), it will be important to determine the pattern of transcription in cells infected with defective virions. Conceivably, the transition from early to late mRNA synthesis (12) could be controlled by the L_1 function.

Co-infection of cells with R_2E virus and defective virus at 39 C results in the production of defectives. The missing L_1 function has been complemented by the mutant. There is, however, virtually no associated growth of the E mutant at 39 C (Table 1). Since growth of both E mutant and defective virus occurs in the co-infected culture at 31 C, the lack of growth of R_2E virus at 39 C is not the result of its inhibition by defectives growing in the same cell, but of lack of complementation of the E function by the defective virus. Thus, complementation by this pair of viruses is asymmetric. Such a situation has been observed with bacteriophage systems (for example, 5 and 9) and with a pair of *ts* mutants of poliovirus (1). Apparently, the L_1 function of reovirus is a *trans*-function; the simplest explanation of this situation is that the L_1 segment codes for a soluble protein that can participate in the replication of either genome of a co-infecting

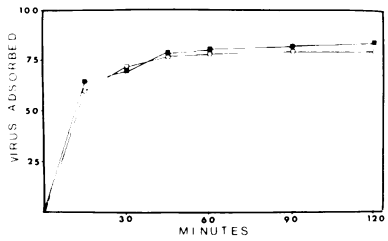

FIG. 8. *Adsorption to L cells of defective virions and R_2 virions. Defective virions were the ^{35}S-labeled, fourth passage progeny of the mixed infection of R_2E virus and R_1d cores. Defective virus and ^3H-labeled R_2 virus, each at a multiplicity of 750 particles per cell, were mixed and allowed to adsorb to L cells at intervals, centrifuged at 600 × g for 10 min, and the radioactivity remaining in the supernatant was determined. These values, and the known amount of each isotope added with the virus, were used to calculate the percentage of each virus adsorbed.* (□) *defective virions;* (■) *R_2 virions.*

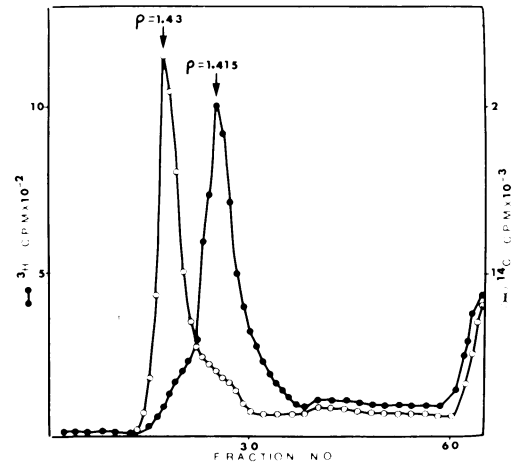

FIG. 9. *Sedimentation in CsCl of cores obtained from the progeny of the mixed infection at 39 C of $R_2B(352)$ and R_1d virions. Cells were co-infected with 10 PFU/ml of R_2B virus and 2 × 10^3 R_1d virions per cell. The virus was labeled with [^3H]uridine during its growth.* (○) *cores obtained from ^{14}C-labeled R_2 virus was added as a density marker;* (●) *cores from the ^3H-labeled progeny of the mixed infection at 39 C.*

pair of viruses. On the other hand, the E function is *cis*-limited and its action is confined to its own genome. The nature of the E function is not yet known. Whereas the E function seems to be involved in a very early step of the infectious cycle (7), it must be expressed after transcription of the parental viral genome because the E mutant can complement R_1d virus. *Cis*-limitation on the E function could be explained if, for example, it were a nondiffusible component of a replicase complex, or if it were a linker protein that held the genomic segments in alignment during replication. Presently there is no evidence for either suggestion.

L_1 has been termed an early viral function (20). R_1d virus should thus be complemented by any class of *ts* mutant that does not have the mutation in the L_1 function or in some earlier function such as the virion transcriptase. Results with R_2A and R_2B class mutants (Fig. 7) show that both complement the defective virions. It is not yet known whether or not the A and B functions are *cis*-limited, but this can be determined by the techniques described in the present paper. Clearly, the defective virions can be used both to elucidate the L_1 function, and to get more information about the nature of the *ts* mutations themselves. For example, it should be fairly easy to determine whether the presently known classes of *ts* mutants have their mutations in *cis*- or *trans*-functions. Methods could probably be developed in which defective virus is used to select for new classes of *ts* mutants. The scope of analyses of this sort would be broadened considerably if defective virus with deletions in other genomic segments could be found, and we are presently searching for such defectives.

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LITERATURE CITED

1. Cooper, P. D. 1965. Rescue of one phenotype in mixed infections with heat-defective mutants of type 1 poliovirus. Virology **25**:431-438.
2. Cross, R. K., and B. N. Fields. 1972. Temperature-sensitive mutants of reovirus type 3: studies on the synthesis of viral RNA. Virology **50**:799-809.
3. Fields, B. N. 1971. Temperature-sensitive mutants of reovirus type 3: features of genetic recombination. Virology **46**:142-148.
4. Fields, B. N., and W. K. Joklik. 1969. Isolation and preliminary genetic and biochemical characterization of temperature-sensitive mutants of reovirus. Virology **37**:335-342.
5. Francke, B., and D. S. Ray. 1972. *Cis*-limited action of the gene-A product of bacteriophage φX174 and the essential bacterial site. Proc. Natl. Acad. Sci. U.S.A. **69**:475-479.
6. Huang, A. S. 1973. Defective interfering viruses, p. 101-117. *In* M. P. Starr, J. L. Ingraham, and S. Raffel (ed.). Annual review of microbiology. Annual Reviews Inc., Palo Alto, Calif.
7. Ito, Y., and W. K. Joklik. 1972. Temperature-sensitive mutants of reovirus. I. Patterns of gene expression by mutants of groups C, D and E. Virology **50**:189-201.
8. Joklik, W. K. 1972. Studies on the effect of chymotrypsin on reovirions. Virology **49**:700-715.
9. Lindahl, G. 1970. Bacteriophage *P2*: replication of the chromosome requires a protein which acts only on the genome that coded for it. Virology **42**:522-533.
10. Millward, S., and M. Nonoyama. 1970. Segmented structure of the reovirus genome. Cold Spring Harbor Symp. Quant. Biol. **35**:773-779.
11. Nonoyama, M., and A. F. Graham. 1970. Appearance of defective virions in clones of reovirus. J. Virol. **6**:693-694.

12. Nonoyama, M., S. Millward, and A. F. Graham. 1974. Control of transcription of the reovirus genome. Nucleic Acids Res. **1**:373-385.
13. Nonoyama, M., Y. Watanabe, and A. F. Graham. 1970. Defective virions of reovirus. J. Virol. **6**:226-236.
14. Prevec, L., Y. Watanabe, C. J. Gauntt, and A. F. Graham. 1968. Transcription of the genomes of type 1 and type 3 reoviruses. J. Virol. **2**:289-297.
15. Schuerch, A. R., and W. K. Joklik. 1973. Temperature-sensitive mutants of reovirus. IV. Evidence that anomalous electrophoretic migration behavior of certain double-stranded hybrid species is mutant group-specific. Virology **56**:218-229.
16. Shatkin, A. J., J. D. Sipe, and P. C. Loh. 1968. Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. J. Virol. **2**:986-991.
17. Smith, R. E., H. J. Zweerink, and W. K. Joklik. 1969. Polypeptide components of virions, top component and cores of reovirus type 3. Virology **39**:791-81.
18. Studier, F. W. 1972. Bacteriophage T7. Science **176**:367-376.
19. Studier, F. W. 1973. Analysis of bacteriophage early RNA's and proteins on slab gels. J. Mol. Biol. **79**:237-248.
20. Watanabe, Y., S. Millward, and A. F. Graham. 1968. Regulation of transcription of the reovirus genome. J. Mol. Biol. **36**:107-123.
21. Zweerink, H. J., M. J. McDowell, and W. K. Joklik. 1971. Essential and nonessential noncapsid reovirus proteins. Virology **45**:716-723.